Morphine postconditioning protects against reperfusion injury: the role of protein kinase c-epsilon, extracellular signal-regulated kinase 1/2 and mitochondrial permeability transition pores

Chen, Zuolei ; Spahn, Donat R ; Zhang, Xuewei ; Liu, Yingzhi ; Chu, Haichen ; Liu, Zhongkai

Abstract: BACKGROUND/AIMS The purpose of this study was to investigate the implications of protein kinase C-epsilon (PKC), Extracellular Signal-regulated Kinase 1/2 (ERK1/2) and mitochondrial permeability transition pore (mPTP) in myocardial protection induced by morphine postconditioning (MpostC). METHODS The isolated rat hearts were randomly assigned into one of eight groups. Hearts in time control (TC) group were constantly perfused for 105min. Hearts in ischemia-reperfusion (I/R) group were subjected to 45 min of ischemia followed by 1 h of reperfusion. MpostC was induced by 10 min of morphine administration at the onset of reperfusion. V1-2 (an inhibitor of PKC) and PD (an inhibitor of ERK1/2) was administered with or without morphine during the first 10 min of reperfusion following the 45 min of ischemia. I/R injury was assessed by functional parameters, creatine kinase-MB (CK-MB) release and infarct size (IS/AAR). Additional hearts were excised at 20 min following reperfusion to detect the membrane-specific translocation of PKC , ERK1/2 phosphorylation, mitochondrial permeability transition (MPT) and cytochrome C (Cyt-c) release. RESULTS MpostC markedly reduced infarct size (IS/AAR), CK-MB release, and improved cardiac function recovery. However, these protective effects were partly abolished in the presence of V1-2 or PD. Compared to TC group, the membrane translocation of PKC , ERK1/2 phosphorylation, mPTP opening, and Cyt-c release were significantly increased in I/R hearts. MpostC further increased the membrane translocation of PKC and ERK1/2 phosphorylation, and significantly inhibited mPTP opening and Cyt-c release. However, those protective effects induced by MpostC were abolished by V1-2 or PD, which, used alone, showed no influence on reperfusion injury. CONCLUSIONS These findings suggest that MpostC protects isolated rat hearts against ischemia-reperfusion injury via activating PKC -ERK1/2 pathway and inhibiting mPTP opening.

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Original Paper

Morphine Postconditioning Protects Against Reperfusion Injury: the Role of Protein Kinase C-Epsilon, Extracellular Signal-Regulated Kinase 1/2 and Mitochondrial Permeability Transition Pores

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Key Words
Morphine • Postconditioning • Reperfusion injury • Protein kinase C epsilon • Extracellular signal-related kinases • Mitochondrial permeability transition pores

Abstract
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and Cyt-c release. However, those protective effects induced by MpostC were abolished by εV_{1-2} or PD, which, used alone, showed no influence on reperfusion injury. ***Conclusions:*** These findings suggest that MpostC protects isolated rat hearts against ischemia-reperfusion injury via activating PKCε-ERK1/2 pathway and inhibiting mPTP opening.

Introduction

We previously demonstrated that administration of morphine immediately at the onset of reperfusion, namely morphine postconditioning (MpostC), reduced the infarct size to an extent similar to morphine preconditioning (MPC) [1], and our recent study further showed that the cardioprotection induced by MpostC is via inhibiting JNK/p38 MAPK and mitochondrial permeability transition pores (mPTP) signaling pathway [2]. Because anisomycin (an activator of JNK/p38 kinases) only partly reversed the infarct-sparing effect of MpostC, it implies that there might be alternative mechanisms involved in MpostC.

One family of signaling proteins commonly linked to the modulation of ischemia–reperfusion injury is protein kinase C (PKC), particularly the PKC isozyme PKCε [3, 4]. Previous research demonstrated that membrane translocation of PKCε was critical to triggering cardioprotective effects in both ischemic preconditioning (preC) [5] and ischemic postconditioning (postC) [6, 7]. Furthermore, PKCε activation is also involved in the cardioprotective effect produced by opioid receptor agonist induced preC [8, 9]. Is PKCε also involved in opioid receptor agonist induced posC? And if it is, what is the potential downstream target of PKCε activation? All these questions are still unclear.

Extracellular signal-regulated kinase (ERK) 1/2 is one important component of reperfusion injury survival kinases (RISK), which are activated at the time of reperfusion and afforded opportunity for protecting the heart against lethal reperfusion-induced injury [10]. Evidence shows that the activation of ERK1/2 was implicated in both preC [11] and postC [12], indicating that preC and postC possibly recruit a common signal pathway during myocardial reperfusion. Furthermore, recent studies suggested that the phosphorylation of ERK1/2 was involved in opioid receptor agonist induced postC [13, 14], but the exact mechanism through which ERK1/2 was activated in opioid-induced postC remains unclear.

It is well known that mPTP plays a paramount role in reperfusion injury [15], and studies showed that both ischemic and anesthetic induced postC afford cardioprotection via inhibiting mPTP opening by activation of ERK1/2 [16, 17]. Therefore, the objective of the present study was to determine whether the RISK pathway (PKCε and ERK1/2) and mPTP were involved in MpostC-induced cardioprotection.

Materials and Methods

All experimental procedures and protocols used in this investigation were reviewed and approved by the Institutional Animal Care and Use Committee of the Affiliated Hospital of Qingdao University and were performed in accordance with the Guide for the Care and Use of Laboratory Animals from the Institute of Laboratory Animals Resources.

Isolated Heart Preparation

Preparation of isolated rat hearts was performed as we previously described [1]. In brief, male Sprague-Dawley rats weighing 180–200 g were anesthetized with an intraperitoneal injection of 40 mg/kg sodium pentobarbital and decapitated. The hearts were rapidly excised, mounted on a Langendorff apparatus, and retrogradely perfused at 100 cm H_2O with Krebs-Ringer's solution (115 mM NaCl, 5 mM KCl, 1.2 mM MgSO4, 1.2 mM KH_2PO_4, 1.25 mM $CaCl_2$, 25 mM $NaHCO_3$, and 11 mM glucose) gassed with 95% O_2-5% CO_2 (pH 7.4, temperature 37°C). A fluid-filled latex balloon was inserted through left atrium and passed through the mitral orifice into the left ventricle. The balloon was connected to a pressure transducer for continuous monitoring of left ventricular developed pressure (LVDP), left ventricular end-diastolic pressure (LVEDP) and heart rate

(HR) via PowerLab Systems (PowerLab/8sp, AD Instruments, Australia). At first, the heart was ongoing a 15-min stabilization period, and any heart showing intractable arrhythmia or low left ventricular systolic pressure (LVSP) < 50 mmHg was excluded from the study.

Experimental Protocols

All hearts were randomly divided into 1 of 8 groups (n = 8, respectively)v(Fig. 1). ①TC group (time control): hearts were constantly perfused with K-R buffer for 105 min; ②I/R group (ischemic control): hearts were subjected to 45 min of ischemia followed by 1 h of reperfusion; ③MpostC group (morphine, 3.0 μmol/L); ④MpostC +$\varepsilon V_{1\text{-}2}$ group: $\varepsilon V_{1\text{-}2}$ (PKCε inhibitor), 1.0 μmol/L]; ⑤$\varepsilon V_{1\text{-}2}$ group; ⑥MpostC +PD group: PD (ERK1/2 inhibitor), 20.0 μmol/L; ⑦PD group; ⑧DMSO group (DMSO, 0.02%). After a 45-min ischemia period, hearts in group ⑤, ⑦ and ⑧ were administered morphine (3.0 μmol/L), $\varepsilon V_{1\text{-}2}$, PD and DMSO respectively for 10 min at the onset of reperfusion, and then were followed by 50 min reperfusion, while hearts in group ④ and ⑥ were treated with morphine (3.0 μmol/L) co-administered with $\varepsilon V_{1\text{-}2}$ and PD for 10 min at the onset of reperfusion respectively, and then were followed by 50 min reperfusion. Additional hearts were used 20 min after reperfusion of the above 8 groups (n = 5, respectively) to detect the membrane-specific translocation of PKCε (group①—⑤), ERK1/2 phosphorylation, mitochondrial permeability transition (MPT) and cytosolic levels of cytochrome C (Cyt-c) (group①—⑧). The concentrations used in this study were based on previous studies [1, 17, 18], and all reagents, unless specified, were obtained from Sigma Chemicals (St. Louis, MO).

Determination of myocardial infarct size and myocardial injury

Myocardial infarct size and myocardial injury were measured as we previously described [1]. In short, infarct size was determined by 2, 3, 5-triphenyltetrazolium chloride (TTC) staining method, and infarct size was determined by dividing the total necrotic area of the left ventricle by the total left ventricular slice area (IS/AAR). In addition, the release of CK-MB, expressed as U/h/g, was measured by collecting total coronary effluent over the 60 min reperfusion period.

Cytosolic and particulate fraction preparation

Cytosolic protein contents and particulate fraction of the myocardium were prepared in accordance with Gao et al. [19]. In brief, frozen samples were homogenized at 0°C-4°C in buffer A consisting of 50 mM Tris-Cl, pH 7.5, 2 mM EDTA, 2 mM EGTA, 1mM dithiothreitol, and inhibitors for protease and phosphatase. These homogenates were centrifuged at 300,000 g for 30 minutes at 4°C in an Optima TLX Ultracentrifuge (Beckman Coulter CO.) to yield the cytosolic fraction. Resuspended in buffer B (buffer A + 0.5% Nonidet P-40), the crude membrane fraction pellet was sonicated and then centrifuged at 300,000 g for 30 minutes at 4°C. The final supernatant was the

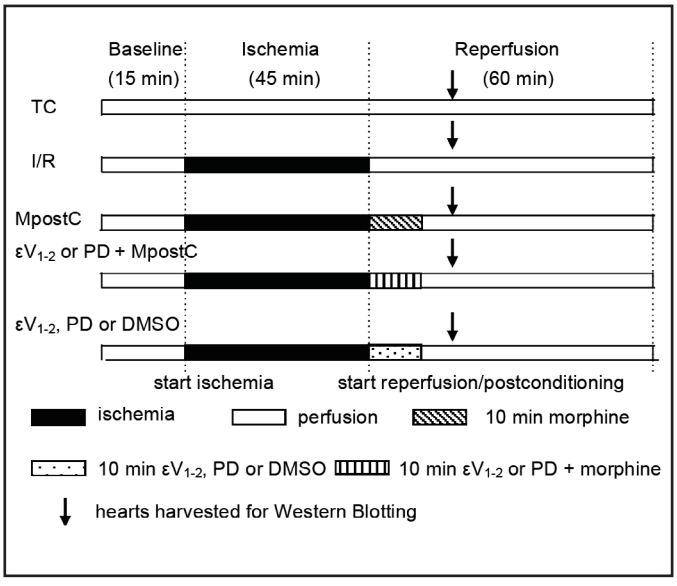

Fig. 1. Scheme of treatment protocols. Heats in TC group were constantly perfused with K-R buffer for 105min. Hearts in other groups were subject to 45 min ischemia followed by 60 min reperfusion. MpostC was performed by a 10-min perfusion of morphine at the onset of reperfusion. $\varepsilon V_{1\text{-}2}$ and PD was administered with or without morphine. Left ventricular tissue samples were collected for Western Blotting and spectrophotometric analysis at 20 min following reperfusion. Infarct size and CK-MB release were measured at the end of reperfusion. TC, time control; I/R, ischemia reperfusion; MpostC, morphine postcondtioning; PD, ERK1/2 inhibitor; DMSO, dimethyl sulphoxide.

particulate fraction. The protein concentrations were measured by BCA protein assay kit (Pierce, Rockford, IL) and using bovine serum albumin standards.

Western blotting analysis

40μg of protein from each sample was loaded per lane in 10% SDS polyacrylamide gel electrophoresis (PAGE) gels. After being electrotransferred and blocked, the membrane was incubated overnight at 4°C with the primary antibody anti-PKCε (1:1000) and a secondary antibody conjugated with horseradish peroxidase (1:1000; Santa Cruz Biotechnology Inc., CA, USA) at room temperature. After washing three times, bands were detected using ECL-plus reagents (Pharmacia Biotech, Piscataway, NJ). To check the slight variation in protein loading between samples, western blot for β-actin was performed as an internal control. The relative optical density of bands from each sample was normalized against that of β-actin, and results were presented as a percentage of TC.

To determine the changes of ERK1/2 phosphorylation, 80μg of protein from the whole tissue homogenate was loaded per lane in 8% SDS-PAGE gel. The primary antibodies specific to ERK1/2 and phosphorylated ERK1/2$^{Thr\text{-}202/\ Tyro\text{-}204}$ were purchased from Santa Cruz (1:1000; Santa Cruz Biotechnology Inc., CA, USA). The phosphorylation levels of ERK1/2 were expressed as a percentage of ERK1/2.

Detection of mPTP opening and Cyt - c release in cytosol

The preparation of mitochondria and cytosolic fractions and detection of mPTP opening and Cyt - c was performed as we previously described [2]. Left ventricular myocardium was homogenized in cold isolation medium, and cell debris was pelleted by centrifuging the homogenate twice for 5 min at 600 g; the supernatant was centrifuged for 10 min at 10 000 g at 4°C, then the pellet was resuspended in isolation buffer and washed twice by recentrifugation at 10 000 g for 10 min at 4°C; the final pellet was resuspended in isolation buffer as a mitochondrial preparation, and the supernatant was centrifuged for 1 h at 100,000 g to obtain cytosolic fraction. The protein concentrations were measured as above described.

Isolated mitochondria (1 mg protein) were resuspended in swelling buffer to a final volume of 2 ml, and incubated at 25°C for 2 min. mPTP opening, induced by 2, 20 and 200 μmol/L $CaCl_2$, was followed by Mitochondrial permeability transition (MPT), and resulted in mitochondrial swelling, and was measured by spectrophotometry (DU800; Beckman Coulter, USA) as a reduction in the optical density at 540nm (OD540) during 5 min (ΔOD/min).

After SDS-PAGE, cytosolic proteins were transferred to a nitrocellulose membrane and incubated with anti-Cytochrome c and β-actin antibodies (mouse polyclonal antibodies diluted 1:200, Santa Cruz Biotechnology, USA) for 6 hours followed by a peroxidase-conjugated secondary antibody. The signal was detected and measured as ascribed above.

Statistical analysis

Data are expressed as mean ± SD. Hemodynamic data were analyzed using two-way repeated measures analysis of variance for time and treatment effects. If an overall significance was found, comparisons between groups were done for each time point using one-way analysis of variance, followed by Tukey *post hoc* testing. For other data, one-way analysis of variance with Tukey *post hoc* testing for multiple comparisons was used, and $P < 0.05$ (two-tailed) was considered statistically significant. ALL statistical analysis was performed with SPSS 19.0 software (SPSS Inc., Chicago, .USA).

Results

Effects on hemodynamics

The baseline hemodynamics were similar among all experimental groups ($P > 0.05$), and all variables remained constant in TC group during the experiment (Table 1). Compared to baseline, the cardiac function in the other seven groups demonstrated deterioration at 10, 30, 60 min of reperfusion, as evidenced by an obvious reduction in CF, HR, and LVDP, and a significant increase in LVEDP ($P < 0.05$). All hemodynamic variables were better in MpostC group than those in I/R group ($P < 0.05$). However, the functional improvements elicited by MpostC were partly reversed by either $\varepsilon V_{1\text{-}2}$ or PD, both of which did not influence

Table 1. Effects of various treatments on hemodynamic parameters. Baseline, 15 min after stabilization; I/R, ischemia reperfusion; HR, heart rate; LVDP, left ventricular developed pressure; LVEDP, left ventricular end-diastolic pressure; MpostC, morphine postconditioning; εV_{1-2}, PKCε inhibitor; PD, ERK1/2 inhibitor; DMSO, dimethyl sulphoxide. Data were presented as mean ± SD (n = 8/group). $^{*}P < 0.05$ vs. baseline (intragroup comparison), $^{\dagger}P < 0.05$ vs. respective value in TC group (intergroup comparison), $^{\S}P < 0.05$ vs. respective value in I/R group (intergroup comparison)

		Reperfusion		
	Baseline	10min	30min	60min
HR, beats/min				
TC	295±12	290±18	292±15	288±13
I/R	298±15	165±15*†	168±18*†	160±12*†
MpostC	298±11	238±16*†§	236±18*†§	230±13*†§
MpostC+ εV_{1-2}	301±12	180±16*†§	190±18*†§	182±15*†§
εV_{1-2}	296±14	165±15*†	166±16*†	162±12*†
MpostC + PD	300 ± 14	187 ± 18*†§	190 ± 20*†§	185 ± 15*†§
PD	301 ± 15	166 ± 16*†	165 ± 12*†	166 ± 15*†
DMSO	295 ± 16	170 ± 18*†	160 ± 14*†	162 ± 13*†
LVDP, mmHg				
TC	120±10	118±15	116±12	115±10
I/R	118±12	54±6*†	60±10*†	58±8*†
MpostC	120±8	90±10*†§	88±8*†§	86±6*†§
MpostC+ εV_{1-2}	121±11	68±11*†§	70±7*†§	68±8*†§
εV_{1-2}	118±10	56±6*†	60±8*†	60±5*†
MpostC + PD	116 ± 12	68 ± 8*†§	69 ± 10*†§	65 ± 5*†§
PD	117 ± 10	54 ± 10*†	61 ± 11*†	56 ± 6*†
DMSO	118 ± 7	59 ± 11*†	60 ± 7*†	58 ± 7*†
LVDEP				
TC	4.1±0.5	4.5±0.5	4.2±0.6	4.6±0.3
I/R	4.2±0.6	42.8±7.0*†	45.1±6.8*†	45.5±6.5*†
MpostC	4.1±0.5	18.6±5.0*†§	18.5±6.5*†§	19.0±5.8*†§
MpostC+ εV_{1-2}	4.3±0.5	32.0±4.5*†§	33.3±4.2*†§	33.0±5.5*†§
εV_{1-2}	4.1±0.3	42.0±5.0*†	45.0±5.8*†	45.2±6.5*†
MpostC + PD	4.6 ± 0.6	32.0.4 ± 6.5*†§	32.0 ± 5.0*†§	33.2 ±4.7*†§
PD	4.4 ± 0.5	41.3 ± 5.8*†	42.4 ± 5.0*†	43.0 ± 4.9*†
DMSO	4.3 ± 0.4	42.5 ± 5.5*†	42.7 ± 5.7*†	43.0 ± 6.0*†

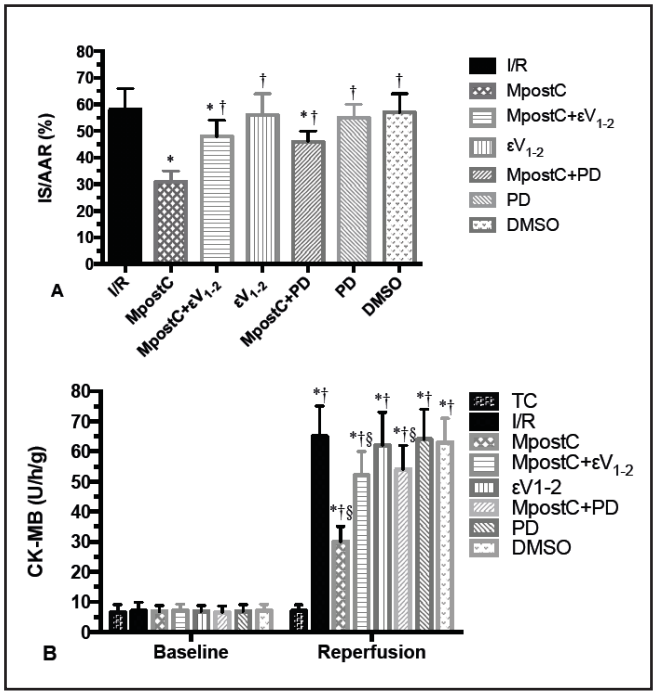

Fig. 2. Effects of various treatments on infarct size (IS/AAR) (A) and CK-MB release (B). Infarct size was expressed as a percentage of the area at risk (IS/AAR). TC group was not included because no obvious necrotic area was identified. TC, time control; I/R, ischemia reperfusion; MpostC, morphine (3.0 μmol/L); εV_{1-2}, 1.0 μmol/L; PD, 20 μmol/L; DMSO, 0.02%. Values were presented as mean ± SD (n = 8/group). $^{*}P < 0.05$ vs. TC, $^{\dagger}P < 0.05$ vs. I/R, $^{\S}P < 0.05$ vs. MpostC.

cardiac recovery when used alone ($P > 0.05$). In addition, DMSO had no effect on cardiac hemodynamics ($P > 0.05$).

Effects on myocardial infarction and CK-MB release

Compared to I/R group, MpostC markedly reduced IS/AAR ($P < 0.05$) (Fig. 2A) and CK-MB ($P < 0.05$) (Fig. 2B). However, the protective effects induced by MpostC were partly reversed by coadministration of either εV_{1-2} or PD ($P < 0.05$), both of which, when given

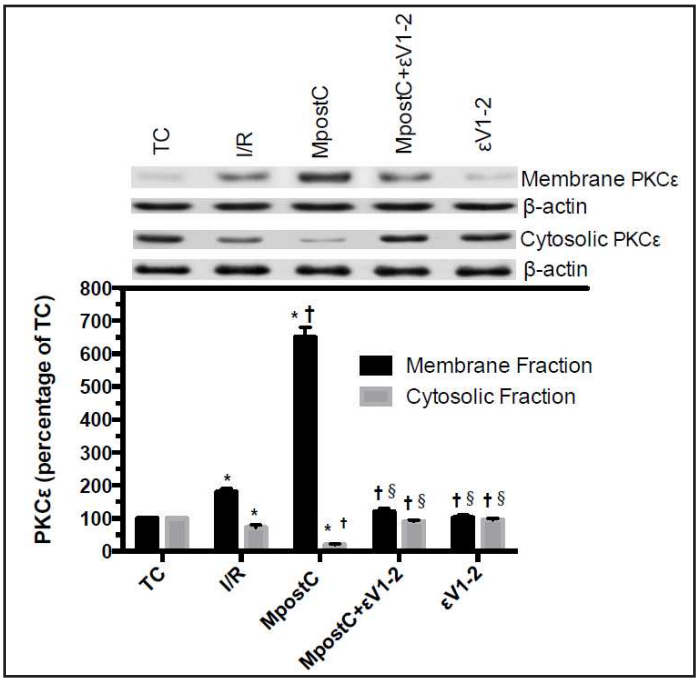

Fig. 3. Effects of various treatments on membrane-specific translocation of PKCε. The upper panel shows representative membrane and cytosolic PKCε of 5 groups. The densities of PKCε were normalized against those of β-actin, and the results were expressed as percentage to TC, as shown in the low panel. TC, time control; I/R, ischemia reperfusion; MpostC, morphine (3.0 μmol/L); $εV_{1-2}$, 1.0 μmol/L. Values were presented as mean ± SD (n = 5/group). * $P < 0.05$ vs. TC, † P < 0.05 vs. I/R, § P < 0.05 vs. MpostC.

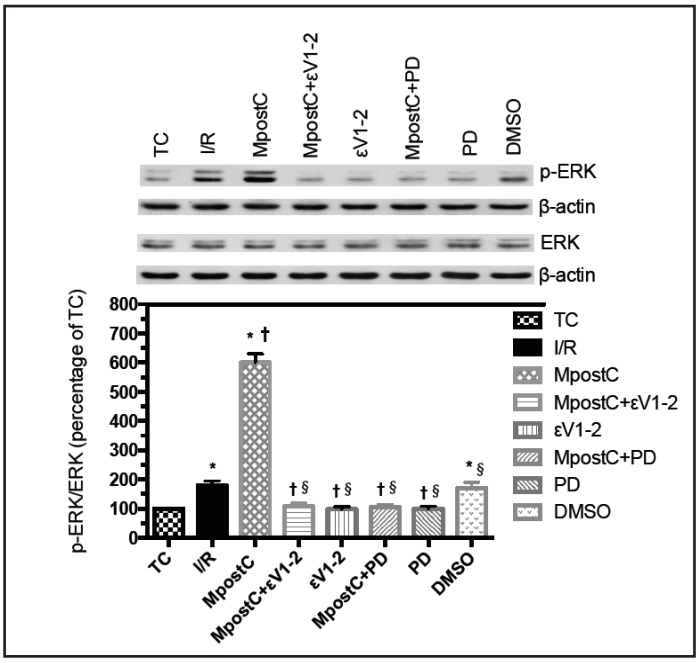

Fig. 4. Effects of various treatments on ERK1/2 phosphorylation. The upper panel shows representative ERK1/2 phosphorylation of each of the eight groups. The densities of phosphorylated ERK1/2 were normalized against total ERK1/2 expression, and the results were expressed as percentage to TC as shown in the lower panel. TC, time control; I/R, ischemia reperfusion; MpostC, morphine (3.0 μmol/L); $εV_{1-2}$, 1.0 μmol/L; PD, 20 μmol/L; DMSO, 0.02%. Values were presented as mean ± SD (n = 5/group). * $P < 0.05$ vs. TC, † P < 0.05 vs. I/R, § P < 0.05 vs. MpostC.

alone, showed no influence on the aspects ($P = 0.12$, $P = 0.10$; $P = 0.10$, $P = 0.08$). DMSO had no effect on IS/AAR and CK-MB release ($P = 0.25$, $P = 0.20$).

Effects on membrane-specific translocation of PKCε

Compared with TC group, the membrane translocation of PKCε from the cytosolic fraction to the particulate was significantly increased in I/R group ($P < 0.05$) (Fig. 3). MpostC further enhanced the membrane translocation of PKCε, when compared to hearts in I/R group ($P < 0.05$). $εV_{1-2}$, administered alone or together with morphine, significantly inhibited membrane translocation of PKCε ($P < 0.05$) (Fig. 3), suggesting that PKCε activation is involved in MpostC.

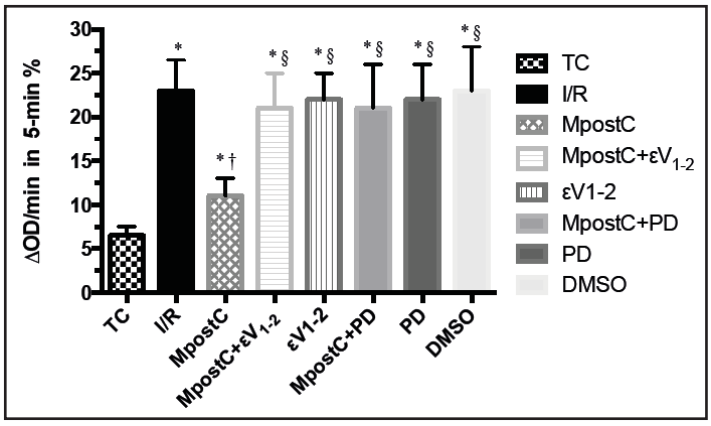

Fig. 5. Effects of various treatments on mitochondrial permeability transition (MPT). MPT was expressed as a reduction in OD540 during 5 min (ΔOD/min). TC, time control; I/R, ischemia reperfusion; MpostC, morphine (3.0 μmol/L); $εV_{1-2}$, 1.0 μmol/L; PD, 20 μmol/L; DMSO, 0.02%. Values were presented as mean ± SD (n = 5/group). * $P < 0.05$ vs. TC, † $P < 0.05$ vs. I/R, § $P < 0.05$ vs. MpostC.

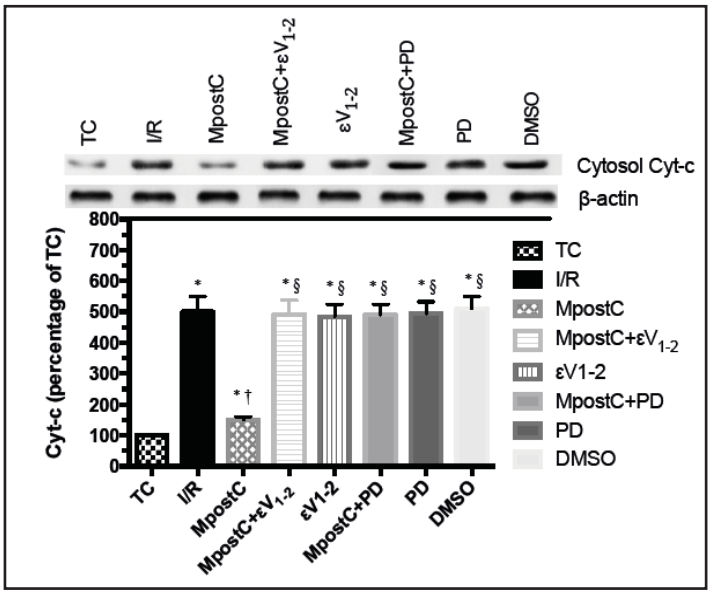

Fig. 6. Effects of various treatments on Cytochrome C (Cyt-c) in cytosol. The upper panel shows representative Cyt-c levels of 5 groups. The densities of Cyt-c were normalized against those of β-actin, and the results were expressed as percentage to TC as shown in the low panel. TC, time control; I/R, ischemia reperfusion; MpostC, morphine (3.0 μmol/L); $εV_{1-2}$, 1.0 μmol/L; PD, 20 μmol/L; DMSO, 0.02%. Values were presented as mean ± SD (n = 5/group). * $P < 0.05$ vs. TC, † $P < 0.05$ vs. I/R, § $P < 0.05$ vs. MpostC.

Effects on ERK1/2 phosphorylation

Compared with TC group, ERK1/2 phosphorylation was significantly increased in I/R group ($P < 0.05$) (Fig. 4). MpostC further enhanced ERK1/2 phosphorylation, when compared to hearts in I/R group ($P < 0.05$). Both $εV_{1-2}$ and PD, administered alone or together with morphine, significantly inhibited ERK1/2 phosphorylation ($P < 0.05$), suggesting that PKCε plays an important role in inducing ERK1/2 phosphorylation either after I/R or MpostC. DMSO did not influence ERK1/2 phosphorylation ($P = 0.22$).

Effects on Ca^{2+} induced mPTP opening

When compared with TC group, ΔOD/min in the I/R group was markedly increased ($P < 0.05$), while hearts in MpostC group have a lower ΔOD/min than those of I/R group (Fig. 5). When co-administered with morphine, either $εV_{1-2}$ or PD abolished the inhibitory effect on ΔOD/min observed with MpostC ($P < 0.05$). In addition, $εV_{1-2}$, PD or DMSO had no influence on the ΔOD/min when used alone ($P = 0.16$, $P = 0.25$, $P = 0.30$).

Effects on cytosolic levels of Cyt–c

The cytosolic level of Cyt-c was significantly increased in I/R group, when compared to TC group ($P < 0.05$) (Fig. 6). Relative to I/R group, MpostC significantly reduced the level of Cyt-c in cytosol ($P < 0.05$). Both $εV_{1-2}$ and PD abolished the inhibitory effect of MpostC on *Cyt–c* release ($P < 0.05$), while $εV_{1-2}$, PD or DMSO had no influence on cytosolic levels of Cyt-c ($P = 0.11$, $P = 0.20$, $P = 0.33$).

Discussion

In the present study, we investigated the roles of PKCε, ERK1/2 and mPTP in morphine-induced postC in the isolated rat heart. The new findings were summarized as follows: (1) Because εV_{1-2} and PD partly abolished the cardioprotection of MpostC, it indicated that both PKCε and ERK1/2 are involved in MpostC; (2) Subsequent western blot analysis showed that MpostC significantly enhanced PKCε membrane translocation and ERK1/2 phosphorylation. In addition, these effects were completely blocked by εV_{1-2}, demonstrating that PKCε might be an upstream regulator of ERK1/2. (3) Because PD abolished the inhibitory effect on mPTP opening induced by MpostC, suggesting that such protective effect occurs downstream of ERK1/2 activation. Taken together, the present study showed that MpostC protects against reperfusion injury via activating PKCε-ERK1/2-mPTP signaling pathway in isolated rat heart.

The activation of PKC is associated with membrane translocation from the cytosol to the particulate fraction of the cell [20], and the activation of PKC, in particular, the PKCε isoform, is pivotal in protecting hearts from ischemia reperfusion injury in preC [3]. Furthermore, studies suggested that the infarct-sparing effect of ischemic postC is also dependent on the activation of PKCε [6, 19]. Therefore, it seems that preC and postC might share some common pathways to protect the heart. Zhang and colleagues showed that remifentanil (an opioid analgesic) mimics cardioprotective effect of ischemic preC via PKC activation [21], while the present study provided new evidence that the activation of PKC isoform ε is involved in cardiprotection induced by MpostC.

As an important reperfusion injury salvage kinase (RISK), ERK1/2 plays a pivotal role in protecting the heart against ischemia reperfusion injury [10, 22], and studies indicated that the protective effects of ischemic and anesthetic postC were conferred by ERK1/2 phosphorylation [12, 17]. The present study demonstrated that MpostC protected the hearts against reperfusion injury through the phosphorylation of ERK1/2, which is consistent with what Ha et al. [13] and Kim et al. [14] suggested using remifentanil postC (an exogenous opioid receptor agonist) strategy. However, the mechanisms through which ERK1/2 is activated in postconditioned hearts are still unclear. The activation of ERK1/2 is regulated by phosphorylation, and this process was shown to be PKC-dependent [23]. Moreover, the PKCε-ERK1/2 signaling cascade was shown to be involved in the protective effect of ischemic preC [11, 24], while the present study further verified that ERK1/2 phosphorylation is also regulated by PKCε translocation in MpostC, which offers new insights into the post-receptor signaling pathway in opioid receptor agonist-induced postC.

On the contrary, it was demonstrated that ERK1/2-dependent activation of PKCε was involved in desflurane preC induced protective effects in *in vivo* rat hearts [25]. Furthermore, Penna et al. suggested that acid postC protected against myocardial I/R injury by activating ERK1/2-PKCε pathways in isolated rat hearts [26]. Though the differences of treatments (preC versus postC), dugs (morphine versus desflurane or acid perfusion), and animal models (*in vitro* versus *in vivo*) might account for the inconsistent observations, we cannot exclude the presumption that there might be an ERK1/2-PKCε positive feedback regulation in postC, namely, that PKCε dependently phosphorylates ERK1/2 while the phosphorylated ERK1/2 in turn activates PKCε because it has been shown that ERK 1/2 is both upstream and downstream of PKC in preC [27]. More evidence is necessary to further verify this presumption.

It is well known that mPTP plays a paramount role in reperfusion injury [15, 28, 29], and it has been strongly implicated as an end-effector in postC [27, 30]. We recently verified MpostC prevents mPTP opening via inhibiting JNK/p38 MAPK activation in isolated rat heats [2]. Given that anisomycin only partly reversed the infarct-sparing effect of MpostC, it implies that there might be alternative mechanisms involved in MpostC. Using the same experimental model, the present findings indicated that activating PKCε–ERK1/2 pathway is an alternative mechanism involved in inhibition of mPTP opening induced by MpostC, which is consistent with what Yao et al. founded using anesthetic-induced postC strategy [17].

So far, the specific mechanism through which ERK1/2 regulates mPTP in myocardium is still unknown. It has been demonstrated that PKCε and ERK1/2 interact and form functional

signaling modules in mitochondria [24], furthermore, studies verified that the active PKCε, designated PKCε1 that is bound to the mitochondrial inner membrane, phosphorylates mK_{ATP} and causes it to open [31]; the resulting increase in K^+ influx with attendant matrix alkalinization then causes increased reactive oxygen species (ROS) production by complex I of the respiratory chain, and the ROS activates a second pool of PKCε, designated PKCε2, which inhibits MPT in a phosphorylation-dependent reaction [32].

The current results must be interpreted within the following limitations. (1) In contrast to an *in vivo* model, isolated hearts have a limited long-term biologic stability and may undergo short confounding ischemic periods during the surgical procedure, which could potentially affect our observation. (2) There is not general agreement concerning the optimal time of reperfusion necessary to assess myocardial infarct size on isolated perfused heart model. In isolated mice heart, Rossello et al. reported that infarct size progressively increases with the duration of both ischemia and reperfusion, and concluded that 180-min reperfusion might be optimal [33]. While in isolated rat heart, Ferrera and colleagues demonstrated that 60-min reperfusion is sufficient to demark infarct zones with no additional infarction occurring with longer periods of reperfusion [34]. In the present study with 45-min ischemia and 60-min reperfusion protocol in isolated rat heart, we cannot completely rule out the possibility that the shorter reperfusion time might be insufficient to wash out LDH and cofactors from necrotic cells to influence the effect of TTC staining when compared to 2-3 h. Taken together, it is necessary to be prudent to explain the present results.

In conclusion, the present study shows MpostC protects isolated rat hearts against ischemia-reperfusion injury via activating PKCε-ERK1/2 pathway and inhibiting mPTP opening, which provides new insights into the post-receptor signal pathways involved in opioid-induced postC in myocardium.

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Disclosure Statement

None.

References

1 Chen Z, Li T, Zhang B: Morphine postconditioning protects against reperfusion injury in the isolated rat hearts. J Surg Res 2008;145:287-294.
2 Chen Z, Zhang X, Liu Y, Liu Z: Morphine Postconditioning Protects against Reperfusion Injury via Inhibiting JNK/p38 MAPK and Mitochondrial Permeability Transition Pores Signaling Pathways. Cell Physiol Biochem 2016;39:61-70.
3 Inagaki K, Churchill E, Mochly-Rosen D: Epsilon protein kinase C as a potential therapeutic target for the ischemic heart. Cardiovasc Res 2006;70:222-230.
4 Wang Y, Men M, Yang W, Zheng H, Xue S: MiR-31 Downregulation Protects Against Cardiac Ischemia/Reperfusion Injury by Targeting Protein Kinase C Epsilon (PKCepsilon) Directly. Cell Physiol Biochem 2015;36:179-190.
5 Duquesnes N, Lezoualc'h F, Crozatier B: PKC-delta and PKC-epsilon: foes of the same family or strangers? J Mol Cell Cardiol 2011;51:665-673.
6 Zatta AJ, Kin H, Lee G, Wang N, Jiang R, Lust R, Reeves JG, Mykytenko J, Guyton RA, Zhao ZQ, Vinten-Johansen J: Infarct-sparing effect of myocardial postconditioning is dependent on protein kinase C signalling. Cardiovasc Res 2006;70:315-324.

7 Zhong GQ, Tu RH, Zeng ZY, Li QJ, He Y, Li S, He Y, Xiao F: Novel functional role of heat shock protein 90 in protein kinase C-mediated ischemic postconditioning. J Surg Res 2014;189:198-206.
8 Wang G-Y, Zhou JJ, Shan J, Wong T-M: Protein kinase C-ε is a trigger of delayed cardioprotection against myocardial ischemia of κ-opioid receptor stimulation in rat ventricular myocytes. J Pharmaco Exp Ther 2001;299:603-610.
9 Miura T, Yano T, Naitoh K, Nishihara M, Miki T, Tanno M, Shimamoto K: δ-Opioid receptor activation before ischemia reduces gap junction permeability in ischemic myocardium by PKC-ε-mediated phosphorylation of connexin 43. Am J Physiol-Heart Circ Physiol 2007;293:H1425-H1431.
10 Hausenloy DJ, Yellon DM: New directions for protecting the heart against ischaemia-reperfusion injury: targeting the Reperfusion Injury Salvage Kinase (RISK)-pathway. Cardiovasc Res 2004;61:448-460.
11 Xuan YT, Guo Y, Zhu Y, Wang OL, Rokosh G, Messing RO, Bolli R: Role of the protein kinase C-epsilon-Raf-1-MEK-1/2-p44/42 MAPK signaling cascade in the activation of signal transducers and activators of transcription 1 and 3 and induction of cyclooxygenase-2 after ischemic preconditioning. Circulation 2005;112:1971-1978.
12 Darling CE, Jiang R, Maynard M, Whittaker P, Vinten-Johansen J, Przyklenk K: Postconditioning via stuttering reperfusion limits myocardial infarct size in rabbit hearts: role of ERK1/2. Am J Physiol-Heart Circ Physiol 2005;289:H1618-1626.
13 Ha JY, Lee YC, Park SJ, Jang YH, Kim JH: Remifentanil postconditioning has cross talk with adenosine receptors in the ischemic-reperfused rat heart. J Surg Res 2015;195:37-43.
14 Kim HS, Cho JE, Hong SW, Kim SO, Shim JK, Kwak YL: Remifentanil protects myocardium through activation of anti-apoptotic pathways of survival in ischemia-reperfused rat heart. Physiol Res 2010;59:347-356.
15 Penna C, Perrelli M-G, Pagliaro P: Mitochondrial pathways, permeability transition pore, and redox signaling in cardioprotection: therapeutic implications. Antioxid Redox Signal 2013;18:556-599.
16 Li H, Wei C, Gao J, Bai S, Li H, Zhao Y, Li H, Han L, Tian Y, Yang G, Wang R, Wu L, Xu C: Mediation of dopamine D2 receptors activation in post-conditioning-attenuated cardiomyocyte apoptosis. Exp Cell Res 2014;323:118-130.
17 Yao YT, Li LH, Chen L, Wang WP, Li LB, Gao CQ: Sevoflurane postconditioning protects isolated rat hearts against ischemia-reperfusion injury: the role of radical oxygen species, extracellular signal-related kinases 1/2 and mitochondrial permeability transition pore. Mol Biol Rep 2010;37:2439-2446.
18 Pravdic D, Sedlic F, Mio Y, Vladic N, Bienengraeber M, Bosnjak ZJ: Anesthetic-induced Preconditioning Delays Opening of Mitochondrial Permeability Transition Pore via Protein Kinase C-ϵ–mediated Pathway. Anesthesiology 2009;111:267-274.
19 Gao J, Guo J, Li H, Bai S, Li H, Wu B, Wang L, Xi Y, Tian Y, Yang G: Involvement of dopamine D2 receptors activation in ischemic post-conditioning-induced cardioprotection through promoting PKC-ε particulate translocation in isolated rat hearts. Mol Cell Biochem 2013;379:267-276.
20 Kraft AS, Anderson WB: Phorbol esters increase the amount of Ca2+, phospholipid-dependent protein kinase associated with plasma membrane. Nature 1983;301:621-623.
21 Zhang Y, G IRWIN M: Remifentanil mimics cardioprotective effect of ischemic preconditioning via protein kinase C activation in open chest of rats. Acta Pharmacol Sin 2005;26:546-550.
22 Zhang L, Wu Y, Li Y, Xu C, Li X, Zhu D, Zhang Y, Xing S, Wang H, Zhang Z, Shan H: Tanshinone IIA improves miR-133 expression through MAPK ERK1/2 pathway in hypoxic cardiac myocytes. Cell Physiol Biochem 2012;30:843-852.
23 Ping P, Zhang J, Cao X, Li RC, Kong D, Tang XL, Qiu Y, Manchikalapudi S, Auchampach JA, Black RG, Bolli R: PKC-dependent activation of p44/p42 MAPKs during myocardial ischemia-reperfusion in conscious rabbits. Am J Physiol 1999;276:H1468-1481.
24 Baines CP, Zhang J, Wang G-W, Zheng Y-T, Xiu JX, Cardwell EM, Bolli R, Ping P: Mitochondrial PKCε and MAPK form signaling modules in the murine heart enhanced mitochondrial PKCε-MAPK interactions and differential MAPK activation in PKCε-induced cardioprotection. Circ Res 2002;90:390-397.
25 Toma O, Weber NC, Wolter JI, Obal D, Preckel B, Schlack W: Desflurane preconditioning induces time-dependent activation of protein kinase C epsilon and extracellular signal-regulated kinase 1 and 2 in the rat heart *in vivo*. Anesthesiology 2004;101:1372-1380.
26 Penna C, Perrelli MG, Tullio F, Angotti C, Pagliaro P: Acidic infusion in early reperfusion affects the activity of antioxidant enzymes in postischemic isolated rat heart. J Surg Res 2013;183:111-118.

27 Cohen MV, Downey JM: Ischemic postconditioning: from receptor to end-effector. Antioxid Redox Signal 2011;14:821-831.
28 Li D, Liu M, Tao TQ, Song DD, Liu XH, Shi DZ: Panax quinquefolium saponin attenuates cardiomyocyte apoptosis and opening of the mitochondrial permeability transition pore in a rat model of ischemia/reperfusion. Cell Physiol Biochem 2014;34:1413-1426.
29 Zhang P, Lu Y, Yu D, Zhang D, Hu W: TRAP1 provides protection against myocardial ischemia-reperfusion injury by ameliorating mitochondrial dysfunction. Cell Physiol Biochem 2015;36:2072-2082.
30 Lim SY, Davidson SM, Hausenloy DJ, Yellon DM: Preconditioning and postconditioning: the essential role of the mitochondrial permeability transition pore. Cardiovasc Res 2007;75:530-535.
31 Costa AD, Garlid KD, West IC, Lincoln TM, Downey JM, Cohen MV, Critz SD: Protein kinase G transmits the cardioprotective signal from cytosol to mitochondria. Circ Res 2005;97:329-336.
32 Costa AD, Jakob R, Costa CL, Andrukhiv K, West IC, Garlid KD: The mechanism by which the mitochondrial ATP-sensitive K^+ channel opening and H2O2 inhibit the mitochondrial permeability transition. J Biol Chem 2006;281:20801-20808.
33 Rossello X, Hall AR, Bell RM, Yellon DM: Characterization of the Langendorff Perfused Isolated Mouse Heart Model of Global Ischemia-Reperfusion Injury: Impact of Ischemia and Reperfusion Length on Infarct Size and LDH Release. J Cardiovasc Pharmacol Ther 2016;21:286-295.
34 Ferrera R, Benhabbouche S, Bopassa JC, Li B, Ovize M: One hour reperfusion is enough to assess function and infarct size with TTC staining in Langendorff rat model. Cardiovasc Drugs Ther 2009;23:327-331.